

AD_____

Award Number: W81XWH-11-1-0145

TITLE: Nerve Degeneration and Regeneration Associated with NF1 Tumors

PRINCIPAL INVESTIGATOR: David F. Muir

CONTRACTING ORGANIZATION: University of Florida
Grinter Hall Gainesville, FL 32611-5500

REPORT DATE: June 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

*Form Approved
OMB No. 0704-0188*

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

1. REPORT DATE June 2013			2. REPORT TYPE Annual			3. DATES COVERED 15 May 2012 - 14 May 2013	
4. TITLE AND SUBTITLE Nerve Degeneration and Regeneration Associated with NF1 Tumors						5a. CONTRACT NUMBER W81XWH-11-1-0145	5b. GRANT NUMBER W81XWH-11-1-0145
						5c. PROGRAM ELEMENT NUMBER	5d. PROJECT NUMBER
							5e. TASK NUMBER
							5f. WORK UNIT NUMBER
6. AUTHOR(S) David F. Muir E-Mail: muir@mbi.ufl.edu						8. PERFORMING ORGANIZATION REPORT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Florida 207 Grinter Hall Gainesville, FL 32611-5500						10. SPONSOR/MONITOR'S ACRONYM(S)	
						11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited							
13. SUPPLEMENTARY NOTES							
14. ABSTRACT Infiltrating peripheral nerve sheath tumors (PNST) are associated with significant neurological deficits and nerve damage. An initial aim of this project is to determine how tumor progression leads to loss of nerve function. A second aim is to determine if nerve damage caused by PNST is reversible and the potential for nerve regeneration after PNST eradication. A third aim is to examine the effects of photodynamic therapy on normal nerve function. Additional aims will test photodynamic therapy as modality for eradication of PNST without incurring substantial collateral damage to functioning nerve. To date we have accomplished the first and second aims and have nearly completed the third. Outcomes for aims 1 and 2 were reported in a previous Annual Report. Our recent work showed that PDT, like most other tissue ablation techniques, has the potential to damage normal peripheral nerves. Therefore, damage to functioning axons is likely when PDT is used for the ablation of tumors growing within nerves, such as PNST. Normal nerves coursing in proximity to tumor targeted for PDT ablation may also be damaged. Importantly, we find that, unlike other cancer therapies, PDT causes an axonometric lesion without significant damage to the nerve sheaths. Consequently, axons will regrow naturally and excellent recovery of function can be expected. Knowing that PDT-induced collateral nerve damage is reversible will have a dramatic impact on therapy selection and on how aggressive the approach can be to achieve tumor free margins. Our next objectives will determine if axonal regeneration and recovery of function can occur after PDT is used to ablate PNST.							
15. SUBJECT TERMS peripheral nerve sheath tumor, neurofibroma, photodynamic therapy, neurological deficit							
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC		
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U	UU	7	19b. TELEPHONE NUMBER (include area code)		

Table of Contents

	<u>Page</u>
Introduction.....	1
Body.....	2
Key Research Accomplishments.....	4
Reportable Outcomes.....	4
Conclusion.....	4
Supporting data.....	6

INTRODUCTION

Infiltrating peripheral nerve sheath tumors (PNST) are associated with significant neurological deficits and defy surgical removal without incurring further nerve damage. PNSTs often cause progressive demyelination, Schwann cell displacement and variable loss of supporting cells and axons within the tumor. An initial objective of this project is to determine how tumor progression leads to loss of nerve function. A second objective is to determine if nerve damage caused by infiltrating PNST is reversible and the potential for nerve regeneration after PNST eradication. Our studies will test the hypothesis that tumor-induced nerve damage and loss of neurological function is reversible. We will determine the potential for both spontaneous and experimentally induced nerve regeneration after tumor eradication. Photodynamic therapy (PDT) is a promising modality for the eradication of PNSTs. Our preliminary studies indicate that PDT effectively kills human NF1 tumor xenografts growing in mouse nerve. However, we found that highly tumoricidal PDT protocols targeted at normal nerves can cause significant nerve damage. Our preliminary studies show the neurological deficits incurred by PDT are transient and recovery of function occurs naturally in mice. We will test the hypothesis that PDT applied to infiltrating PNSTs can be highly tumoricidal without substantial permanent collateral damage to normal nerve.

BODY

OBJECTIVE 1: Determine the regenerative potential of nerves damaged by peripheral nerve sheath tumors.

Task 1: Examine PNST-induced damage to nerve: [months 1-6].

PROGRESS

Task 1 was completed (see Progress Report for Y1).

Task 2: Evaluate nerve regeneration after immunorejection of PNST: [months 3-12]

PROGRESS

Task 2 was completed (see Progress Report for Y1).

OBJECTIVE 2: Determine the mechanisms and pharmacodynamics of PDT induced nerve damage.

Task 1: Test various drug-light intervals in PDT applied to normal nerve and evaluate nerve damage: [months 12-16]

PROGRESS

PDT is a binary treatment modality that involves systemic administration of a photosensitizer agent followed by activation of the agent by focal irradiation with light. The photosensitizer-light interval is an important determinant of photosensitizer distribution and therefore potential phototoxicity required for tumor cell death. After systemic injection the agent will rapidly accumulate in vascular elements. Therefore, a

short drug-light interval is particularly effective at disruption of tumor vasculature (and normal tissue vasculature as well). Longer drug-light intervals allow clearing of the agent from the endothelium and pronounced accumulation in tumor and normal cells. Both mechanisms (disruption of tumor vasculature with ensuing anoxia and direct cellular phototoxicity) achieve dramatic tumor regression. Various photosensitizer-light intervals were tested for PDT applied to normal nerves in mice. Three days after PDT equivalent loss of nerve function was observed for all intervals tested (Table 1). Histology of the treated nerves revealed vascular damage, hemorrhage and blood flow stasis in larger vessels around the nerve and capillaries within the nerve (Fig. 1). These results indicate that in the mouse sciatic nerve LS11 levels remained cytotoxic (after light activation) in the blood and/or vascular elements 1 hour after injection and for at least 6 hours thereafter. In addition, for all drug-light intervals cell death was evident throughout the nerve segment exposed to PDT (Fig. 2). This indicates that LS11 rapidly permeated the nerve and was taken up by supportive cells (Schwann cells, fibroblasts, etc.) around the nerve fibers. PDT applied to the nerve resulted in focal damage to nerve fibers and supportive cells, however, as expected, the neurons themselves (e.g., dorsal root ganglia) remained viable and therefore capable of regrowing axons back through the nerve tissue exposed to PDT (see Progress Report for Y1). Drug-light intervals of one to six hours are the therapeutic window in cancer treatments. These results indicate that when using a highly tumoricidal dose of LS11 the drug-light interval is not a parameter for minimizing collateral nerve damage with PDT. Future work will determine if this remains true with lower, but still tumoricidal, doses of LS11.

Task 2: Test various drug-light intervals in PDT applied to normal nerve and evaluate nerve regeneration: [months 14-20]

PROGRESS

As stated above, after PDT there was a rapid and complete loss of sensory and motor functions mediated by the sciatic nerve. However, preliminary studies show that the loss of function was transient and that the damaged nerves regenerated naturally. This aim will test if recovery of function and nerve regeneration is better or worse with different drug-light intervals. A second set of mice was treated with various LS11 PDT drug-light intervals. As above, all showed immediate loss of sciatic nerve function. Function testing over a 12-week recovery period and data analyses is underway, but not yet complete. Observations thus far suggest recovery of function occurs after PDT with various drug-light intervals. Further testing and quantitation is required to determine if there are differences in the level of function recovery achieved in the different PDT conditions.

OBJECTIVE 3: Determine the tumoricidal effects of LS11-PDT applied to intraneuronal tumor xenografts.

PROGRESS

This Aim will determine the parameters for an effective PDT applied to infiltrating, intraneuronal PNST xenografts. Various drug-light intervals in LS11-PDT will be tested on PNST xenografts. Three days after PDT, mice will be terminated and tumors examined for

extent and distribution of cell death by TUNEL staining. The goal of these tests is to determine the extent and distribution of tumor and normal neural cell death.

An important variable in these studies is the extent of tumor growth, that is, whether the tumor partially or completely occupies the nerve (and destroys it). We have established methods to monitor tumor growth and nerve destruction along with protocols to achieve tumor growth that replicates early and advanced disease (see Progress Report for Y1, Figs. 1 and 2). Numerous experiments were performed to determine the tumor kill with LS11-PDT applied to intraneuronal tumor xenografts. Our PDT is applied to tumors using the photosensitizer LS11 at 5 mg/kg and photoactivation by LED irradiation (664nm wavelength; 200 Joules/cm). Drug-light intervals of 0.5 to 6 hours will be tested. Thus far we have performed PDT using a 1-hour interval on tumors at various stages of progression. At 6 weeks after tumor initiation we found tumor cells partially occupy nerve and observed the onset of nerve destruction by invading tumor (Fig. 3A). Tumors at this stage cause minor functional deficit of the nerve. PDT applied to 6-week tumors (that partially occupy the nerve) resulted in quantitatively complete tumor cell death (Fig. 3B).

At 8 weeks or more after initiation tumors fully occupy the nerve around the site of injection and greatly expand the tissue structure. These tumors destroy and appear to consume all nerve elements resulting in a complete loss of nerve continuity and function (Fig. 4A). PDT applied to >8-week tumors (that fully occupy the nerve) resulted in quantitatively complete tumor cell death (Fig. 4B). Taken together, these findings indicate that LS11-PDT is fully effective in eradicating nerve tumors at various stages of progression.

OBJECTIVES FOR THE NEXT YEAR

No changes in the Objectives are anticipated in the third year of this project.

OBJECTIVE 3: Determine the tumoricidal effects of LS11-PDT applied to intraneuronal tumor xenografts.

Task 1: Test various drug-light intervals in PDT applied to xenograft tumors and evaluate tumor kill: [months 20-24].

Task 2: Determine the most tumoricidal mode of PDT, vascular versus cell targeting: [months 20-26].

OBJECTIVE 4: Determine the regenerative potential of nerves after tumoricidal PDT treatment.

Task 1: Evaluate long-term regression of nerve sheath tumor xenografts after PDT: [months 26-36].

Task 2: Evaluate long-term effects of tumoricidal PDT on nerve regeneration and recovery of function: [months 26-36].

KEY RESEARCH ACCOMPLISHMENTS

1. Determined that when using a highly tumoricidal dose of LS11 the drug-light interval is not a parameter for minimizing collateral nerve damage with PDT.
2. Observed recovery of function occurs after PDT with various drug-light intervals.
3. Determined that LS11-PDT is fully effective in eradicating nerve tumors at various stages of progression.

REPORTABLE OUTCOMES

1. Animal model for application of LS11-PDT for eradication of nerve tumors
2. Graduate student J. Graham awarded fellowship from Clinical and Translational Science Institute.
3. Submitted grant application to the Florida Biomedical Research Program in Cancer based on work supported by this award.

CONCLUSIONS

Our work tested that hypothesis that tumor-induced nerve damage and loss of neurological function is reversible. Findings indicate that this is true only in the formative stages of tumor growth associated with low-level functional deficits. Tumor progression leads to

increasing and permanent loss of nerve function. Function deficits persist even after tumor eradication. Results indicate that the tumor destroys the nerve, ablates supporting cells and replaces nerve structure with an impenetrable fibrotic mass. We found that the neurons remain viable but fail to regrow axons into the fibrotic mass (even when devoid of tumor cells). This has several implications for managing PNST. Most often the treatment of PNST involves a "wait and see approach" when symptoms are minor and there is little loss of function. This approach is contraindicated by our findings. More disturbing is that substantial tumor damage to nerve may not be reversible even after effective tumor eradication. The main conclusion of our findings is that eradication of well-established tumors does not induce or support nerve regeneration.

LS11-PDT causes immediate nerve damage and loss of function. This outcome occurs at various drug-light intervals (1 to 6 hours). These results indicate that when using a highly tumoricidal dose of LS11 the drug-light interval is not a parameter for minimizing collateral nerve damage with PDT. However, initial results suggest that at various drug-light intervals PDT-induced nerve damage is reversible and recovery of function will occur without surgical intervention.

LS11-PDT effectively eradicates PNST xenografts in the mouse sciatic nerve. Complete tumor kill is achieved for tumors at various stages of progression. Results indicate that early stage tumors (that incur low to moderate nerve damage) can be effectively killed without a good prognosis for recovery of nerve function. LS11-PDT effectively kills advanced stages of PNST. Advanced PNST destroys the nerve region it occupies and creates a significant loss of nerve continuity. It remains to be determined if the nerve can regenerate axons and reinstate continuity leading to recovery of function.

SUPPORTING DATA

Table 1. Effects of various drug-light intervals on PDT induced nerve damage.

Drug-light interval	Function deficit	Vascular disruption	Axonal degeneration	Supportive cell death
1 hour	100%	complete	widespread	widespread
6 hours	100%	complete	widespread	widespread

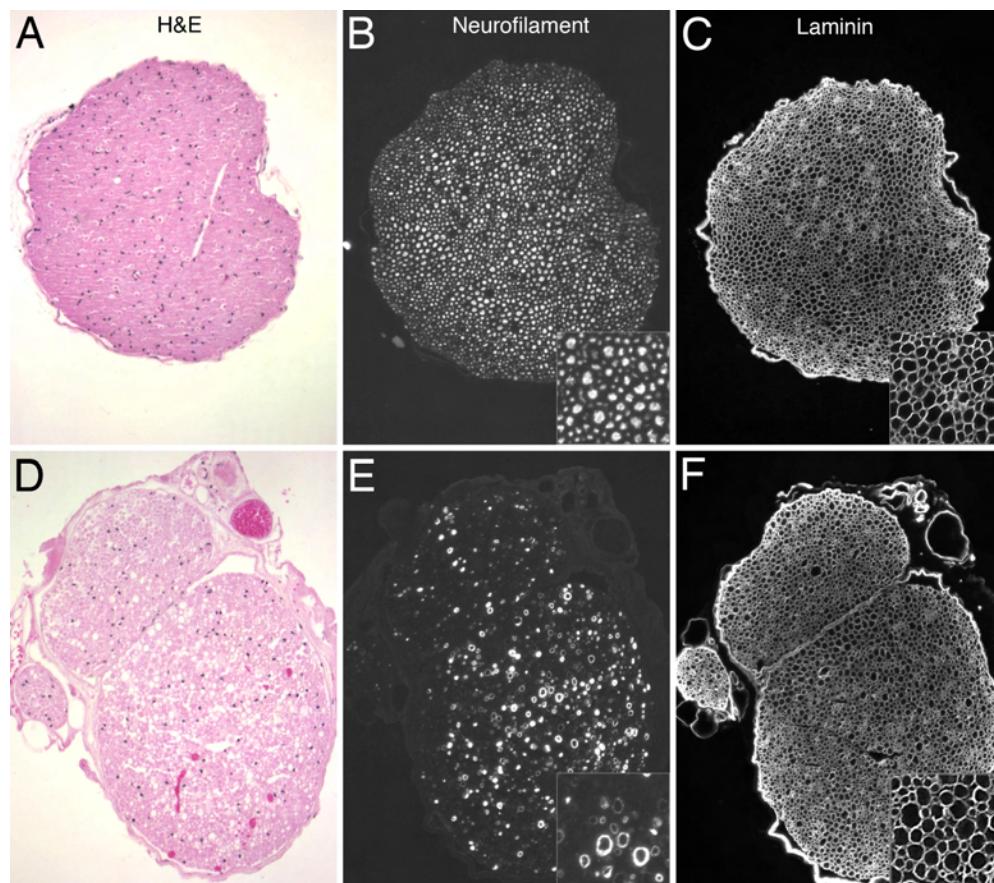


Fig. 1. PDT applied to normal sciatic nerves in mice. Panels A-C show untreated nerve morphology and panels D-F show nerve treated with PDT. H&E staining (A, D) revealed vascular disruption, hemorrhages and swelling after PDT. Neurofilament immunolabeled axons (B, E) were highly disrupted by PDT, as indicated by the appearance of swollen rings (E, inset). Laminin immunolabeling for basement membranes (C, F) indicated the nerve sheaths remained intact.

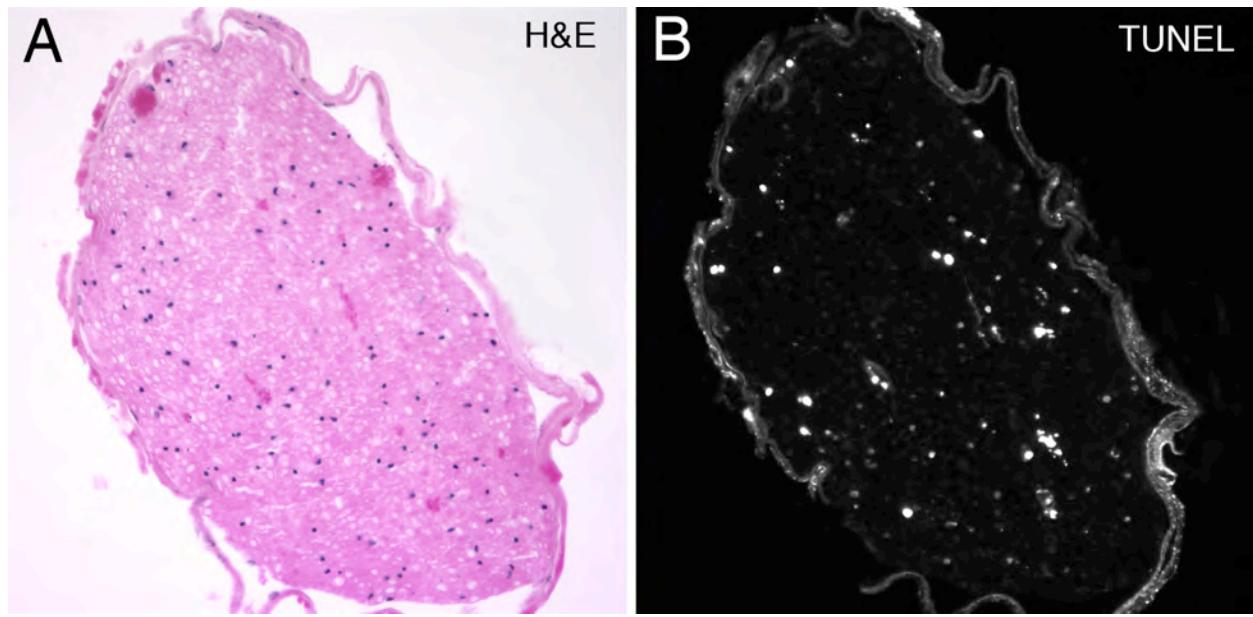


Fig. 2. PDT applied to normal sciatic nerves in mice. Representative photomicrographs of nerves treated with PDT using drug-light intervals of 1 to 6 hours. (A) H&E staining revealed vascular disruption, hemorrhages and swelling after PDT. Blood cells appear dark pink and nuclei of supportive cells appear blue. (B) TUNEL staining for apoptotic cells (bright white) showed PDT at drug-light intervals of 1 to 6 hours caused widespread death of supportive cells within the nerve.

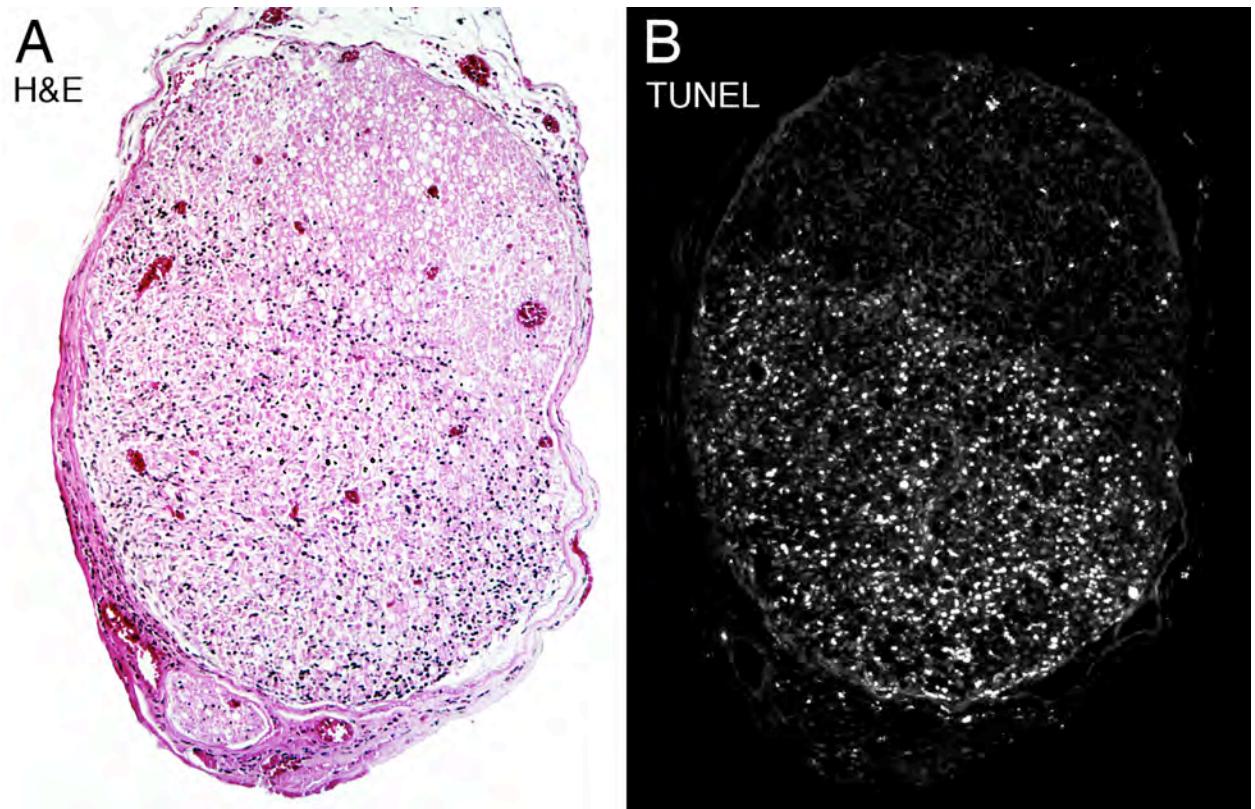


Fig. 3. Representative photomicrographs of early stage tumor xenografts in the sciatic nerves in mice after LS11-PDT. (A) H&E staining revealed tumor hypercellularity (cell nuclei appear blue) vascular disruption, hemorrhages (blood cells appear dark pink) and edema after PDT. (B) TUNEL staining for apoptotic cells (bright white) showed PDT caused extensive death of tumor and neural cells within the area of treated nerve.

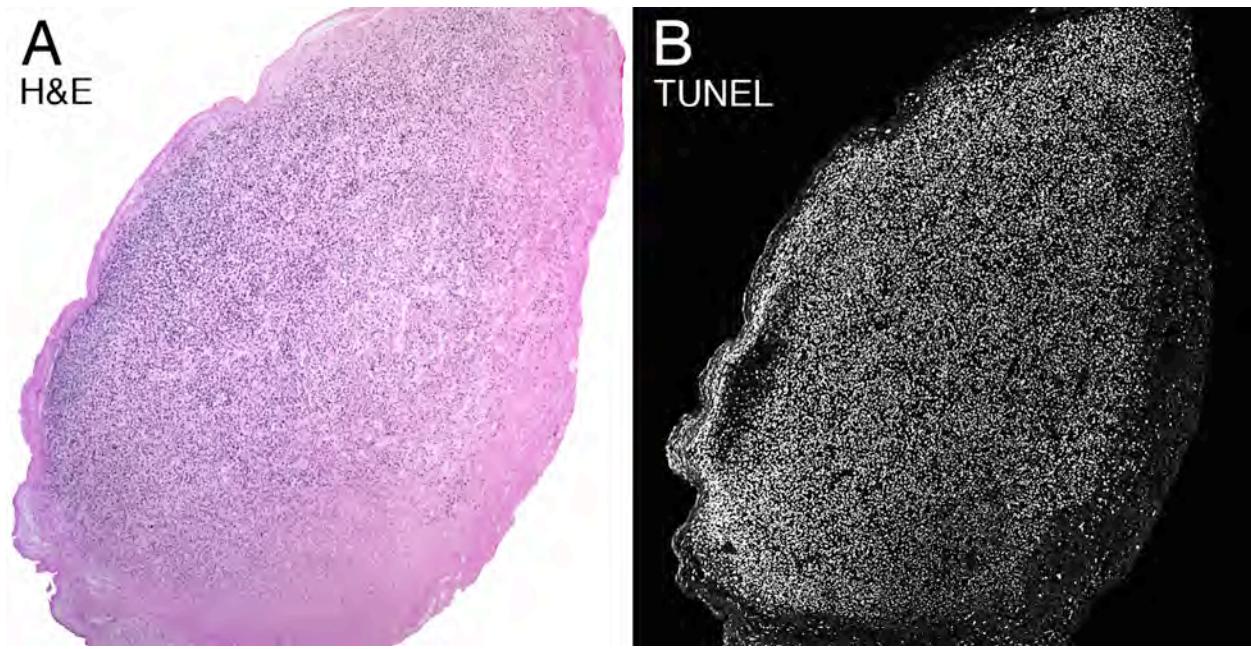


Fig. 3. Representative photomicrographs of late stage tumor xenografts in the sciatic nerves in mice after LS11-PDT. (A) H&E staining revealed a large hypercellular tumor mass (cell nuclei appear blue) and the absence of all nerve elements and structure. The tumor is encapsulated by fibrotic tissue (pink). (B) TUNEL staining for apoptotic cells (bright white) showed PDT caused extensive death of tumor cells within the treated area.